FTO (human intracellular) ELISA Kit

(Catalog #K4921-100; 100 assays; Store kit at 4°C)

I. Description:

FTO, Fat mass-and obesity-associated gene, was discovered as a responsible gene causing the mouse 'fused toes' mutation. The predicted 502-amino acid Fto protein has a calculated molecular mass of 58 kD and contains an N-terminal bipartite nuclear localization signal. FTO is widely expressed in a variety of human tissues, with highest levels in brain and pancreatic islets. Bioinformatics analysis indicates that FTO shares sequence motifs with iron- and 2-oxoglutarate (2OG)-dependent oxygenases. The FTO (human) (IntraCellular) ELISA Kit is to be used for the *in vitro* quantitative determination of human FTO in cell lysates or cell-based assays (screening). This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human FTO in cells. A monoclonal antibody specific for FTO has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, FTO is recognized by the addition of a purified polyclonal antibody specific for FTO (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit IgG (Detector) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of FTO in the samples. This ELISA is specific for the measurement of natural and recombinant human FTO. It does cross-react with human adiponectin, human RBP4, human Nampt, human vaspin, human progranulin, human resistin, human clusterin, human GPX3, human sirtuin 1, human IDO, human IL-33, human ANGPTL3, human ANGPTL4, human FGF21, mouse progranulin, mouse ANGPTL3, mouse leptin, rat Nampt. The assay range is 0.156 – 10 ng FTO/ml and a detection limit of 50 pg/ml (based on adding two standard deviations to the mean value of the (50) zero standards).

II. Kit Contents:

Component	100 Assays	Part Number
Pre-coated Microtiter Plate	1 ea (12 x 8 well strips)	K4921-100-1
Wash Buffer (10X)	50 ml	K4921-100-2
Diluent (5X)	50 ml	K4921-100-3
Lysis Buffer (10X)	12 ml	K4921-100-4
Detection Antibody	12 ml	K4921-100-5
Detector 100X (Hrp conjugated anti-IgG)	150 µl	K4921-100-6
Human FTO Standard (lyophilized, 20 ng)	1 vial	K4921-100-7
Human FTO QC Sample (lyophilized)	1 vial	K4921-100-8
TMB Substrate Solution	12 ml	K4921-100-9
Stop Solution	12 ml	K4921-100-10
Plate Sealers	3 each	K4921-100-11

III. Storage Conditions:

Reagents must be stored at 2 - 8°C when not in use. Bring reagents to room temperature before use. Do not expose reagents to temperatures greater than 25°C.

IV. Assay Procedure (Read the ENTIRE Protocol Before Proceeding)

1. Test Samples/Standards/QC Sample: (We recommend these be run in duplicate)

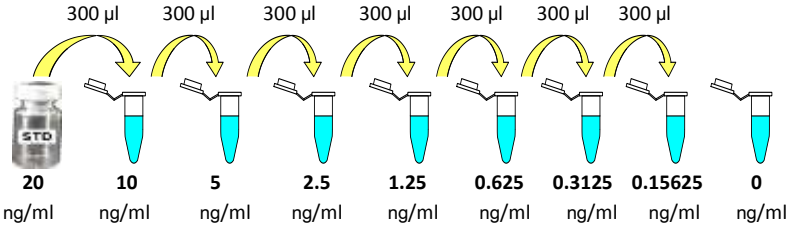
a) **Cell Lysates**: Grow cells to 90% confluency. Scrap cells off the plate and transfer to an appropriate tube. Keep on ice and microcentrifuge at 1,200 rpm for 5 min at 4°C. Remove supernatant, rinse cells once with ice-cold PBS. Remove PBS and add 200 µl ice-cold 1X Lysis Buffer supplemented with 1 mM phenyl methylsulfonyl fluoride (PMSF) to ten million cells. Incubate on ice for 30 min. Microcentrifuge at 12,000 rpm for 5 min at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Use freshly prepared cell lysate samples. **Note**: Cell lysates have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use. As starting point 1/10 to 1/1000 dilutions are recommended.

b) **QC Sample**: Reconstitute human FTO QC Sample with 1 ml of dH_2O. Mix the QC Sample to ensure complete reconstitution. Allow to sit for a minimum of 15 min. The QC Sample is ready to use-do not dilute it (refer to the C of A for current QC Sample concentration).

c) **Standards**: Reconstitute human FTO Standard with 1 ml of dH_2O to produce a stock solution (20 ng/ml). Mix the Stock solution to ensure complete reconstitution. Allow to sit for a minimum of 15 min. The reconstituted standard should be aliquoted and stored at -20°C.

d) Prepare Standard Curve using 2-fold serial dilutions with 1X Diluent:

To obtain	Add	Into
10 ng/ml	300 µl of FTO (20 ng/ml)	300 µl of 1X Diluent
5 ng/ml	300 µl of FTO (10 ng/ml)	300 µl of 1X Diluent
2.5 ng/ml	300 µl of FTO (5 ng/ml)	300 µl of 1X Diluent
1.25 ng/ml	300 µl of FTO (2.5 ng/ml)	300 µl of 1X Diluent
0.625 ng/ml	300 µl of FTO (1.25 ng/ml)	300 µl of 1X Diluent
0.3125 ng/ml	300 µl of FTO (0.625 ng/ml)	300 µl of 1X Diluent
0.15625 ng/ml	300 µl of FTO (0.3125 ng/ml)	300 µl of 1X Diluent
0 ng/ml	300 µl of 1X Diluent	Empty tube



2. Reagent Preparation: (Prepare just the appropriate amounts for the assay)

a) **1X Wash Buffer:** Dilute 10X Wash Buffer 1: 9 with dH_2O to obtain 1X Wash Buffer.
b) **1X Diluent:** Dilute 5X Wash Buffer 1: 4 with dH_2O to obtain 1X Diluent.
c) **1X Lysis Buffer:** Dilute 10X Lysis Buffer 1: 9 with dH_2O to obtain 1X Lysis Buffer.
d) **1X Detector**: Dilute 100X Detector 1: 99 with 1X Diluent to obtain 1X Detector.
e) **Detection Antibody & TMB Substrate Solution**: Ready to use. Warm to room temp before use.

Note: The diluted Detector must be used within 1 hr of preparation

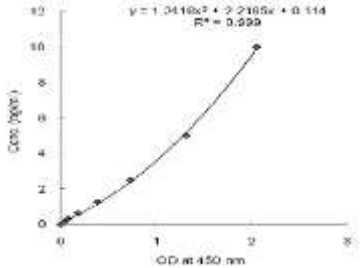
3. Assay Protocol:

a) Determine the number of 8-well strips needed for assay and insert them into the frame for current use. The extra strips should be resealed in the foil pouch and can be stored at 4°C for up to 1 month.
b) Add 100 µl of the Standards, Samples and QC Sample into the appropriate wells in duplicate.
c) Cover plate with plate sealer and incubate for 1 hr at 37°C.
d) Aspirate and wash x 3 with 300 µl of 1X Wash Buffer.
e) Add 100 µl Detection Antibody to each well and tap gently on the side of the plate to mix.
f) Cover plate with plate sealer and incubate for 1 hr at 37°C.
g) Aspirate and wash x 3 with 300 µl of 1X Wash Buffer.
h) Add 100 µl of the 1X Detector to each well.
i) Cover plate with plate sealer and incubate for 1 hr at 37°C.
j) Remove plate from 37°C, aspirate and wash x 5 with 300 µl of 1X Wash Buffer.
k) After last wash, tap inverted plate on a stack of paper towels. Complete removal of liquid is essential for good performance.
l) Add 100 µl of the TMB Substrate Solution to each well.
m) Allow the color to develop at room temperature in the dark for 10 min.
n) Stop the reaction by adding 100 µl of Stop Solution to each well.

o) Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
Caution: Stop Solution is a Corrosive Solution
p) Measure the OD at 450 nm in an ELISA plate reader within 30 min.

3. Calculations:

a) Average the duplicate readings for each Standard, QC Sample and Test Sample and subtract the average blank value (obtained with the 0 ng/ml point).
b) Generate a Standard Curve by plotting the average absorbance on the horizontal (X) axis vs. the corresponding concentration (µg /ml) on the vertical (Y) axis. (See Typical Data below)
c) Calculate the Test Sample FTO concentrations by interpolation of the Standard Curve regression curve as shown below in the form of a quadratic equation.
d) If the Test Samples were diluted, multiply the interpolated values by the dilution factor to calculate the corrected human FTO concentrations.



VI. Performance Characteristics:

1. Intra-assay Precision: (4) samples of known concentrations of human FTO were assayed in replicates (6) times to test precision within an assay.

Samples	Mean (ng/ml)	SD	CV (%)	n
THP-1 cells	31.976	1.789	5.585	6
Molt4 cells	262.277	6.146	2.343	6
A549 cells	96.285	1.569	1.629	6
HepG2 cells	130.960	2.823	2.156	6

2. Inter-assay Precision: (3) samples of known concentrations of human FTO were assayed in (4) separate assays to test precision between assays.

Samples	Mean (ng/ml)	SD	CV (%)	n
Molt4 cells	226.599	20.899	9.223	4
A549 cells	92.102	8.076	8.768	4
HepG2 cells	99.771	4.978	4.989	4

3. Recovery: Different human cell lysates were spiked with known concentrations of human FTO and the recoveries averaged 100 % (range from 95 % to 105 %)

Samples	Average (%)	Range (%)
THP-1 cells	95.416	95 - 105
A549 cells	102.444	95 - 105
HepG2 cells	103.941	95 - 105

Technical Hints and Limitations:

- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

Troubleshooting:

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

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